# PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/87, 15/12, A61K 48/00 C07K 15/00	A1	(11) International Publication Number: WO 92/19749 (43) International Publication Date: 12 November 1992 (12.11.92)

(21) International Application Number: PCT/US92/03639

(22) International Filing Date: 1 May 1992 (01.05.92)

(30) Priority data:
695,598   3 May 1991 (03.05.91)   US

(71) Applicants: THE BOARD OF REGENTS ACTING FOR AND ON BEHALF OF THE UNIVERSITY OF MICHIGAN [US/US]; Ann Arbor, MI 48109 (US). UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). ALBERT EINSTEIN COLLEGE OF MEDICINE, A DIVISION OF YESHIVA UNIVERSITY [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US).

(72) Inventors: WILSON, James, M. ; 3686 River Pines Drive, Ann Arbor, MI 48103 (US). GROSSMAN, Mariann ; 834 West Huron Street, #1, Ann Arbor, MI 48103 (US). WU, Catherine, H. ; 15 Rundelane, Bloomfield, CT 06002 (US). CHOWDHURY, Namita, Roy ; 139 Woodhollow Lane, New Rochelle, NY 10804 (US). WU, George, Y. ; 15 Rundelane, Bloomfield, CT 06002 (US). CHOWDHURY, Jayanta, Roy ; 139 Woodhollow Lane, New Rochelle, NY 10804 (US).

(74) Agents: DeCONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TARGETED DELIVERY OF GENES ENCODING CELL SURFACE RECEPTORS

(57) Abstract

Molecular complexes for targeting a gene encoding a cell surface receptor to a specific cell in vivo and obtaining expression of the gene and insertion of the gene-encoded receptor in the cell membrane. An expressible gene encoding a desired cell surface receptor is complexed with a carrier of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used in gene therapy to selectively transfect cells in vivo to provide for production, membrane insertion and function of a cellular surface receptor.

-1-

TARGETED DELIVERY OF GENES
ENCODING CELL SURFACE RECEPTORS

Background of the Invention

Familial hypercholesterolemia (FH) is an
inherited disease in humans, caused by a deficiency
of low-density lipoprotein (LDL) receptors. FH is
05   associated with hypercholesterolemia and premature
development of coronary heart disease. J.L.
Goldstein and M.S. Brown in The Metabolic Basis of
Inherited Disease (C.R. Scriver, A.L. Beaudet, W.S.
Sly and D. Valle eds.) McGraw-Hill, New York, Sixth
10   edition, pp. 1215-1250. Current therapies for FH
primarily attempt to decrease serum LDL cholesterol
by increasing hepatic expression of the LDL
receptor. J.L. Goldstein and M.S. Brown in The
Metabolic Basis of Inherited Disease supra. This
15   has been accomplished in homozygous patients by
orthotopic liver transplantion from donors who
express normal levels of LDL receptor. Short term
metabolic efficacy in the animal model for FH, the
Watanabe Heritable Hyperlipidemic rabbit (WHHL), has
20   been demonstrated by transplanting allogenic
wild-type hepatocytes or WHHL hepatocytes genetically

corrected ex vivo with recombinant retr virus s.
J.M. Wilson et al. Proc. Natl. Acad. Sci. 85:4421
(1988) and J.M. Wilson et al. Proc. Natl. Acad. Sci.
87:8437 (1990).

05   The clinical utility of ex vivo gene therapy in
the liver may be limited, in part, by the morbidity
of the invasive procedures used to harvest and
transplant hepatocytes. Delivery of genes to
hepatocytes and other cells in vivo would be of value
10 in treating FH and other acquired and inherited
diseases.

Summary of the Invention
        This invention pertains to a soluble molecular
complex for targeting a gene encoding a cell surface
15 receptor, such as the LDL receptor, to a specific
cell in vivo and obtaining expression of the gene by
the targeted cell. The molecular complex comprises
an expressible gene encoding a desired cell surface
receptor complexed with a carrier which is a
20 conjugate of a cell-specific binding agent and a
gene-binding agent. The cell-specific binding agent
is specific for a cellular surface structure,
typically a receptor, which mediates internalization
of bound ligands by endocytosis, such as the
25 asialoglycoprotein receptor of hepatocytes. The
cell-specific binding agent can be a natural or
synthetic ligand (for example, a protein, poly-
peptide, glycoprotein, etc.) or it can be an
antibody, or an analogue thereof, which specifically
30 binds a cellular surface structure which then
mediates internalization of the bound complex. The
gene-binding component of the conjugate is a compound

such as a polycation which stably complexes the gene
under xtracellular conditions and r leases the gene
under intracellular conditions so that it can
function within the cell.

05   The complex of the gene and the carrier is
stable and soluble in physiological fluids. It can
be administered in vivo where it is selectively taken
up by the target cell via the surface-structure-
mediated endocytotic pathway. The incorporated gene
10   is expressed and the gene-encoded receptor is
processed and inserted into the cell membrane of the
transfected cell.

          The soluble molecular complex of this invention
can be used to specifically transfect cells in vivo
15   to provide for expression of a desired cell surface
receptor. This selective transfection is useful for
gene therapy and in other applications which require
selective genetic alteration of cells to produce a
desired surface receptor. In gene therapy, a normal
20   gene can be targeted to a specific cell to correct or
alleviate an inherited or acquired abnormality
involving a cell surface receptor, such as familial
hypercholesterolemia, caused in part by a defect in
the LDL receptor gene.

25   Brief Description of The Figures
          Figure 1 shows the structure of the LDL receptor
expression vector p9-12alb(h)LDLR. The vector
contains the structural gene for human LDL receptor
driven by rat albumin promoter and mouse albumin
30   enhancer sequences.

-4-

Figur 2 shows that the LDLR-complex is primarily targeted to the liver. ^{125}I uptake by various organs was determined following injection of radiolabeled LDLR-complex.

05   Figure 3 shows the cellular distribution of the LDLR-complex which indicates the complex is predominantly taken up by hepatocytes.

Figure 4A shows DNA blot hybridization analysis of total cellular DNA which indicates that the

10 LDLR-complex remains intact extracellularly and delivers functional DNA.

Figure 4B shows RNase protection analysis which confirms the presence of recombinant human LDL receptor transcripts in the liver.

15   Figure 5 shows the results of a two treatment crossover study measuring total serum cholesterol to confirm the presence of human LDL receptor.

Detailed Description of the Invention

A soluble, targetable molecular complex is used

20 to selectively deliver a gene encoding a cell surface receptor to a target cell or tissue in vivo. The molecular complex comprises the receptor-encoding gene to be delivered complexed to a carrier made up of a binding agent specific for the target cell and a

25 gene-binding agent. The complex is selectively taken up by the target cell and the gene-encoded receptor is expressed, processed, and inserted into the cellular membrane.

The gene, generally in the form of DNA, encodes

30 the desired cell surface receptor. Typically, the gene comprises a structural gene encoding the receptor in a form suitable for processing by the

target cell. For example, the gene enc des
appropriate signal sequences which direct processing
and membrane insertion of the receptor. The signal
sequence may be the natural sequence of the receptor
05 or exogenous sequences. The structural gene is
linked to appropriate genetic regulatory elements
required for expression of the gene-encoded receptor
by the target cell. These include a promoter and
optionally an enhancer element operable in the target
10 cell. The gene can be contained in an expression
vector such.as a plasmid or a transposable genetic
element along with the genetic regulatory elements
necessary for expression of the gene and production
of the gene-encoded product.

15   The carrier component of the complex is a
conjugate of a cell-specific binding agent and a
gene-binding agent. The cell-specific binding agent
specifically binds a cellular surface structure which
mediates internalization by, for example, the process
20 of endocytosis. The surface structure can be a
protein, polypeptide, carbohydrate, lipid or
combination thereof. It is typically a surface
receptor which mediates endocytosis of a ligand.
Thus, the binding agent can be a natural or synthetic
25 ligand which binds the receptor. The ligand can be a
protein, polypeptide, glycoprotein or glycopeptide
which has functional groups that are exposed
sufficiently to be recognized by the cell surface
structure. It can also be a component of a
30 biological organism such as a virus, cells (e.g.,
mammalian, bacterial, protozoan) or artificial
carriers such as liposomes.

The binding agent can also be an antibody, or an
analogue of an antibody such as a singl chain
antibody which binds the cell surface structure.

Ligands useful in forming the carrier will vary

05 according to the particular cell to be targeted. For
targeting hepatocytes, glycoproteins having exposed
terminal carbohydrate groups such as asialoglyco-
protein (galactose-terminal) can be used, although
other ligands such as polypeptide hormones may also

10 be employed. Examples of asialoglycoproteins include
asialoorosomucoid, asialofetuin and desialylated
vesicular stomatitis virus. Such ligands can be
formed by chemical or enzymatic desialylation of
glycoproteins that possess terminal sialic acid and

15 penultimate galactose residues. Alternatively,
asialoglycoprotein ligands can be formed by coupling
galactose terminal carbohydrates such as lactose or
arabinogalactan to non-galactose bearing proteins by
reductive lactosamination.

20   For targeting the molecular complex to other
cell surface receptors, other types of ligands can be
used, such as mannose for macrophages, mannose-6-
phosphate glycoproteins for fibroblasts, intrinsic
factor-vitamin B12 for enterocytes and insulin for

25 fat cells. Alternatively, the cell-specific binding
agent can be a receptor or receptor-like molecule,
such as an antibody which binds a ligand (e.g.,
antigen) on the cell surface. Such antibodies can be
produced by standard procedures.

30   The gene-binding agent complexes the gene to be
delivered. Complexation with the gene must be
sufficiently stable _in vivo_ to prevent significant
uncoupling of the gene extracellularly prior to

internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene is released in functional form. For example, the complex can be
05 labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic attraction between the gene-binding agent and the gene provides extracellular stability and is releasable under intracellular conditions.
10   Preferred gene-binding agents are polycations that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but
15 releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine. Other noncovalent bonds that can be used to releasably link
20 the expressible gene include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing
25 biotinylated nucleotides.
    The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be
30 formed with a water soluble carbodiimide as described by G. Jung et al. Biochem. Biophys. Res. Commun. 101:599-606 (1981). An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the
particular cell-specific binding agent and
gene-binding agent used to form the carrier.
Reaction conditions can be designed to maximize
05 linkage formation but to minimize the formation of
aggregates of the carrier components. The optimal
ratio of cell-specific binding agent to gene-binding
agent can be determined empirically. When poly-
cations are used, the molar ratio of the components
10 will vary with the size of the polycation and the
size of the gene. In general, this ratio ranges from
about 10:1 to 1:1, preferably about 5:1. Uncoupled
components and aggregates can be separated from the
carrier by molecular sieve chromatography.

15   The gene encoding the desired cell surface
receptor can be complexed to the carrier by a
stepwise dialysis procedure. In a preferred method,
for use with carriers made of polycations such as
polylysine, the dialysis procedure begins with a 2M
20 NaCl dialyzate and ends with a 0.15M NaCl solution.
The gradually decreasing NaCl concentration results
in binding of the DNA to the carrier.

The molecular complex can contain more than one
copy of the same gene or one or more different
25 genes. Preferably, the ratio of polynucleotide to
the carrier is from about 1:5 to 5:1, preferably
about 1:2.

The molecular complex of this invention can be
administered parenterally. Preferably, it is
30 injected intravenously. The complex is administered
in solution in a physiologically acceptable vehicle.

Cells can be transfected in vivo for transient
expr ssion and production of the gene pr duct. For
prolonged expression and production, the gene can be
administered repeatedly. Alternatively, the

05 transfected target cell can be stimulated to
replicate by surgical or pharmacological means to
prolong expression of the incorporated gene. See,
for example, U.S. Patent Application Serial No.
588,013, filed September 25, 1990, the teachings of

10 which are incorporated by reference herein.

The method of this invention can be used in gene
therapy to selectively deliver a gene encoding a cell
surface receptor to a target cell in vivo for
expression. A gene encoding a desired cell surface

15 receptor can be targeted to a cell which normally
expresses the receptor but which lacks the capacity
to produce the receptor or produces an insuffient
amount of the receptor because of an acquired or
inherited defect. For example, a normal gene can be

20 targeted to a specific cell to correct or alleviate a
metabolic or genetic abnormality caused by an
inherited or acquired defect in a corresponding
endogenous gene encoding a cell surface receptor.
Alternatively, the gene can be delivered to a cell

25 which does not normally express the surface receptor
to confer a new function upon the cell.

The cell surface receptor can be a receptor for
a natural ligand such as a metabolite, a hormone, a
growth factor, a cytokine, an ion (ion transport

30 protein), a virus, or a protozoan. It can also be a
receptor that mediates cell-cell interaction.

In _vivo_ gen transfer has several potential advantages over organ/cell transplantation in the treatment of metabolic diseases of the liver. One advantage is that the therapeutic gene is expressed
05 _in situ_ in a cell and organ that has not been manipulated _ex vivo_. In addition, the capacity of this approach to reconstitute hepatic gene expression is theoretically greater than the capacity of cellular therapies which are usually limited by the
10 number of cells that will engraft. Finally, the therapeutic gene can be delivered to the appropriate cell in a noninvasive way with little apparent morbidity.

Familial hypercholesterolemia is an inherited
15 disease in humans caused by a deficiency in the receptor for LDL. In a preferred embodiment, the gene encoding the human LDL receptor is complexed to a conjugate of an asialoglycoprotein and a polycation. The resulting soluble complex is
20 administered parenterally to the individual afflicted with the LDL receptor deficiency in amounts sufficient to selectively transfect cells and to provide sufficient production of the cell surface receptor to attain normal levels of serum
25 cholesterol. DNA encoding human LDL receptor is described in U.S. Patent 4,745,060, the contents of which are incorporated herein by reference.

-11-

This inventi n is illustrated further by the following Exemplificati n.

Exemplification

05   An asialoglycoprotein-polycation conjugate consisting of asialoorosomucoid (ASOR) coupled to polylysine, was used to form a soluble DNA complex capable of specifically targeting hepatocytes via asialoglycoprotein receptors present on these cells. The DNA comprised a plasmid, p9-12alb(h)LDLR,

10   containing the structural gene for the human LDL receptor driven by rat albumin promoter and mouse albumin enhancer elements.

Animals

The efficacy of intravenous administration of

15   the LDLR-complex was studied in an animal model of familial hypercholesterolimia, the Watanabe Heritable Hyperlipidemic (WHHL) rabbit. WHHL rabbits were derived from mating homozygous LDL-receptor deficient rabbits and were purchased from Dr. Mahlan at New

20   York University. Wild-type New Zealand White (NZW) rabbits were purchased from Dutchland Farms (Denver, PA). Animals were maintained on a Purina laboratory rabbit chow and weighed 2-3 kg at the time of experimentation. Phlebotomies were performed at 3 to 4

25   PM. Experiments were conducted in accordance with the guidelines of the Committee on Use and Care of Animals from the University of Michigan and Albert Einstein College of Medicine.

-12-

Construction of DNA Vector

A vect r capabl of expressing n rmal human LDL
receptor was constructed (p9-12alb(h)LDLR) for _in
vivo_ gene transfer experiments in WHHL rabbits
05   (Figure 1). Transcriptional elements from the mouse
albumin gene were used to drive expression of a
full-length cDNA for the human LDL receptor. L.E.
Babiss et al. Proc. Natl. Acad. Sci. 83:6504 (1986);
C.A. Pinkert et al. Genes Dev. 1:268 (1987); K.S.
10   Zaret et al. Proc. Natl. Acad. Sci. 85:9076 (1988);
and R.S. Herbat et al. Proc. Natl. Acad. Sci. 86:1553
(1989).

The expression vector was constructed in a
single three-part ligation using fragments that were
15   cloned in a directional manner (Figure 1). The three
fragments used in this construction are as follows:
Fragment A is an XbaI to BglII fragment (3.6 kb) of
plasmid MTEV.JT (provided by J. Trill) which contains
several functional elements including a 231 base pair
20   (bp) fragment of genomic DNA spanning the polyadenyl-
ation signal of the bovine growth hormone gene,
ß-lactamase and the prokaryotic origin of replication
from PUC 19, and a eukaryotic transcriptional unit
expressing xanthine-guanine phosphoribosyltransferase
25   (XGPRT); Fragment B includes sequences spanning an
enhancer located 5' to the mouse albumin gene (-12 to
-9 kb) excised on an Eco RV to BglII fragment and
fused in reverse orientation to sequences spanning
the mouse albumin promoter (-282 to +21). L.E.
30   Babiss et al. Proc. Natl. Acad. Sci. 83:6504 (1986);
C.A. Pinkert et al. Genes Dev. 1:268 (1987); K.S.
Zaret et al. Proc. Natl. Acad. Sci. 85:9076 (1988);
and R.S. Herbat et al. Proc. Natl. Acad. Sci. 86:1553

(1989). Th 5' end of the chimeric fragment is formed by the natural BglII site of the albumin enhancer (-9 kb) while the 3' end of the fragment contains a synthetic SalI site attached to position

05   +22 of the albumin promoter sequence; and Fragment C is a SalI to XbaI fragment (3.0 kb) derived from a previously published retroviral vector LTR-LDLR. J.M. Wilson et al. Proc. Natl. Acad. Sci. 85:4421 (1988). The LTR-LDLR vector includes the entire cDNA

10   for human LDL receptor (2.6 kb) along with 430 bp of additional 3' sequence derived from the retroviral genome (nucleotides 7816 to 8113 of the Moloney murine leukemia virus genome; see C. Van Beveren et al. in RNA Tumor Viruses (R. Weiss, N. Teich, H.

15   Varmus, and J. Coffin eds.) Cold Spring Harbor Lab., Cold Spring Harbor N.Y., 2nd Ed., pp. 766-783 (1985) for numbering). The 5' end of the LDL receptor cDNA was converted to a SalI site in preparation for this construction.

20   LDL receptor sequences in p9-12alb(h)LDLR were replaced with sequences encoding the prokaryotic gene chloroamphenicol acetyltransferase (CAT) to generate a vector called p9-12albCAT (provided by S. Camper). This vector was used as a negative control in

25   metabolic experiments based on the assumption that CAT expression does not specifically affect intracellular hepatic cholesterol metabolism. DNA/protein complexes were synthesized with either p9-2alb(h)LDLR (LDLR-complex) or p9-12albCAT

30   (CAT-complex) (See below).

Construction of Gene Carrier and Gene Carrier Complexes

A high affinity ligand for the asialoglycoprotein receptor, asialoorosomucoid

05 (ASOR), was covalently attached to polylysine to produce a gene carrier useful in the study of the organ and cellular distribution of DNA/protein complex uptake in vivo. In order to introduce a radioisotope into the DNA/protein complex without

10 perturbing its structural configuration LDLR-complexes were synthesized with ^{125}I-labeled ASOR. The gene carriers were prepared as described previously. G.Y. Wu et al. J. Biol. Chem. 264:16985 (1989).

15   Briefly, human orosomucoid isolated from pooled human plasma, was desialyted with neuraminidase and subsequently coupled to poly L-lysine (MW=59,000), using 1-ethyl(-3-)-3-dimethyl amino propyl carbodiimide. The ASOR/poly L-lysine conjugate was

20 purified by molecular sieve chromatography as described previously and complexed to plasmid DNA using an agarose gel retardation assay to determine optimal conjugate to DNA ratios for each plasmid. G.Y. Wu et al. J. Biol. Chem. 264:16985 (1989).

25 Large scale complexes were made by successive stepwise dialysis of conjugate-DNA mixtures 18 hours each against 2 M, 1.5 M, 1.0 M, 0.5 M. and finally 0.2 M NaCl. Final samples were filtered through 0.45 μ membranes and checked by agarose gel electro-

30 phoresis for the presence of protein/DNA complex and the absence of free DNA. ASOR was labeled with ^{125}I (G.Y. Wu and C.H. Wu J. Biol. Chem. 262:4429 (1987); and G.Y. Wu and C.H. Wu Biochemistry 27:887 (1988)),

conjugated to poly L-lysine, and the radiolabeled
ASOR/poly L-lysine conjugate was complex d with
p9-12alb(h)LDLR plasmid as described above.

Targeting of the LDLR-Complex to the Liver

05   Animals were anesthetized with ketamine HCl (40
mg/kg) and xylazine (10 mg/kg) in preparation for the
experiment. Radiolabeled LDLR-complexes were
injected into the marginal ear vein of New Zealand
White (NZW) or WHHL adult rabbits (2 to 3 kg) which
10   were euthenized and exanguinated 10 minutes later.
Individual organs were harvested and analyzed for
incorporation of radioactivity (Figure 2).
DNA/protein complex was rapidly cleared from the
plasma and primarily taken up by the liver (85% of
15   total recovered radioactivity) after 10 minutes.
Organ distribution of uptake was shown to be
independent of the phenotype of the recipient animal
(NZW, heterozygous WHHL, and homozygous WHHL) and the
dose of injected complex (from 0.17 mg to 4 mg of DNA
20   in a complex).

The average recovered radioactivity for the four
animals presented in Figure 2 was estimated to be 99
+/-10 percent (mean +/- 1 S.D., N=4). Data are
presented as % recovered radioactivity in individual
25   organs including liver, spleen, lung, heart, kidney,
and blood. (A) Tracer quantities of ASOR labeled
complex (0.17 mg of DNA; specific activity = 2 x 10^6
cpm/µg) into a WHHL rabbit (homozygous). (B) Tracer
quantities of ASOR labeled complex into a WHHL rabbit
30   (heterozygous). (C) Tracer quantities of ASOR
labeled complex (0.17 mg of DNA; specific activity =
2 x 10^6 cpm/µg) into a NZW rabbit. (D) Therapeutic

quantities of ASOR labeled c mplex (4.0 mg of DNA;
specific activity = 0.85 x 10⁵ cpm/μg) into a WHHL
rabbit (homozygous).

Cellular Distribution of the LDLR-Complex

05   Although the hepatocyte is the predominant cell
type in the liver and the likely target for gene
transfer, nonparenchymal cells of the liver, such as
endothelial cells and kupffer cells, could potential-
ly serve as alternative targets. To visualize the
10   cellular distribution of LDLR-complex, rabbits were
injected with radiolabeled LDLR-complex as described
above. Ten minutes after injection, the portal vein
was cannulated, and the liver was perfused with 154
mM NaCl for 5 minutes followed by Karnovsky's
15   fixative for 10 minutes. R. St. Hilaire et al. Proc.
Natl. Acad. Sci. 80:3797 (1983). Random blocks of
liver were cut on ice to 2 - 5 mm³ cubes and were
kept in fresh fixative for 2 hours prior to transfer
to a 0.2 M sodium bicarbonate, pH 7.4, overnight at
20   room temperature. Blocks were embedded in Epox 812
(W.O. Dobbins III in Diagnostic Electron Microscopy
(B.F. Trump and R.T. Jones eds.) Wiley, New York,
Vol. 1, pp. 253-339) and 1 μm sections were cut and
mounted on polylysine-coated slides which were dipped
25   in NTB3 nuclear emulsion. After exposure for 2 - 3
weeks at 4° C, slides were developed with Kodak D-19,
and stained with 0.5% toluidine blue in 1% sodium
benzoate (See Figure 3). Panel A: Analysis of liver
tissue from a WHHL that received ASOR labeled complex
30   (4.0 mg of DNA; specific activity = 0.85 x 10⁵
cpm/μg). Panel B: Analysis of liver tissue from a
WHHL rabbit that was injected with saline. In each
case, magnification is 400x.

Radioactive signal, detected as silv r grains,
was 100-fold over that observed in unlabel d liver
tissue (Figure 3). The majority of this signal
(>90%) was seen as discrete grains located over
05 hepatocytes (Figure 3A). Radioactive signal was
infrequently seen over kupffer cells as aggregates or
discrete grains (Figure 3A). This nonparenchymal
cell uptake may represent larger forms of the
DNA/protein complex that have been phagocytized by
10 kupffer cells. These studies indicate that the
DNA/protein complex is rapidly taken up by
hepatocytes after injection into peripheral blood.

Intracellular Distribution of the LDLR-Complex
         The intracellular fate of the recombinant gene
15 was studied in WHHL rabbits after administration of
unlabeled LDLR-complex. Animals were euthanized 10
minutes, 4 hours, and 24 hours after in vivo gene
transfer and liver tissue was characterized with
respect to the abundance and structural integrity of
20 the recombinant gene as well as the level of
recombinant derived RNA.
         DNA Blot Hybridization Studies:
         Total cellular DNA isolated from liver of WHHL
rabbits was analyzed for the presence of
25 p9-12alb(h)LDLR DNA sequences by blot hybridization
(Figure 4A). J.M. Wilson et al. Proc. Natl. Acad.
Sci. 87:8437 (1990). DNA was restricted with Bam HI
to excise a 1.4 kb fragment from the plasmid,
fractionated by agarose gel electrophoresis, and
30 transferred to Zetabind. Filters were hybridized
with a vector specific restriction fragment (Hind III
to Eco RI fragment of 3Z-env) that had been 32p

-18-

labeled to high specific activity. J.M. Wilson et
al. Proc. Natl. Acad. Sci. 85:4421 (1988). Samples
include control WHHL DNA (10 μg) mixed with the
equivalent of 100 copies/cell (750 pg; lane '100c'),
05   10 copies/cell (75 pg; lane '10c'), and 1 copy/cell
(7.5 pg; lane '1c') of p9-12alb(h)LDLR plasmid DNA.
Additional DNA samples (10 μg) are from WHHL rabbits
injected with LDLR-complex and harvested 10 minutes
(lane '10 min'), 4 hours (lane '4 hr'), and 24 hours
10   (lane '24 hr') later. The filter was exposed to film
for 45 minutes (top panel) and 24 hours (bottom
panel). Molecular weight markers are noted in base
pairs along the left border.

The results indicate that liver tissue harvested
15   10 minutes after injection of the LDLR-complex
demonstrated very high levels of the intact fragment
along with some partially degraded plasmid DNA;
comparison to plasmid controls indicated that this
tissue contained approximately 5,000 to 10,000 copies
20   of plasmid per cell. This estimate of gene targeting
is in agreement with the level of gene delivery to
the liver expected from the amount of DNA injected
(this estimate is based on the injection of 4 mg of
plasmid (10 kb in size) into a 2-3 kg rabbit that has
25   $2 - 4 \times 10^{10}$ hepatocytes). Analysis of livers
harvested at later time points revealed ongoing
degradation of the plasmid and a progressive decline
in the abundance of the intact plasmid from 100
copies/cell at 4 hours to 1 copy/cell at 24 hours.
30   These experiments indicate that the LDLR-complex
remains intact in the blood and capable of efficient
delivery of large quantities of DNA to the liver.
The internalized plasmid DNA is eventually degraded.

RNase Protction Assays:

Liver tissues were analyzd fr the presence and abundance of recombinant human LDL receptor transcripts using a quantitative RNase protection
05 assay. J.M. Wilson et al. Proc, Natl, Acad, Sci, 87:8437 (1990). RNA derived from the p9-12alb(h)LDLR vector was detected with an antisense RNA probe that is synthesized from the previously described vector 3Z-env. J.M. Wilson et al. Proc, Natl, Acad, Sci,
10 87:8437 (1990). This RNA probe is complementary to vector specific sequences in the 3' untranslated region of the recombinant transcript. RNase protection of the resulting duplex produces a protected fragment of 172 bp (Figure 4B). Antisense
15 RNA that specifically detects endogenous WHHL LDL receptor RNA was used as an internal control in each assay.

The transcription vector used to synthesize the RNA probe (3Z-wLDLR) was constructed in the following
20 manner. A restriction fragment from a WHHL LDL receptor cDNA clone, spanning the Sma I site at position 211 to the Nar I site at 514, was isolated (See T. Yamamoto et al. Science 232:1230 (1986) for numbering). The Nar I site was converted to a Hind
25 III site with synthetic linkers and the revised fragment was ligated with the Hind III to Sma I backbone fragment of pGEM3Zf(+) (Promega). Endogenous WHHL LDL receptor RNA is detected with the 3Z-wLDLR probe in RNase protection assays as a 80 bp
30 band. Transcription vectors were linearized with Eco RI (3Z-env) or Mnl I (3Z-wLDLR) and used as templates in transcription reactions according to the recommendations of the manufacturer (Promega). RNA

-20-

probes wer gel purifi d prior t us . M.H. Finer in
Methods in Molecular Biology, Vol. 7: Gene Transfer
and Expression Techniques (E.J. Murray and J.M.
Walker eds.) The Humana Press Inc., Clifton, NJ, pp.
05 1-15 (1990).

        Total cellular RNA prepared from liver was
hybridized with equal quantities of 3Z-env and
3Z-wLDLR probe (5×10^5 cpm of each probe per assay)
and analyzed for protection to digestion with RNase
10 A.   J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437
(1990). Samples were electrophoresed through a 6%
polyacrylamide/urea denaturing gel; a representative
autoradiograph is presented. Radioactivity in the
resulting bands was quantified with a Beta Scope 630
15 (Betagen, Waltham MA). The following samples were
analyzed. Lane '3T3' contains RNA (100 µg) from a
control WHHL supplemented with RNA (100 ng) from a
fibroblast cell line (NIH3T3) that produces
recombinant LDL receptor transcripts containing
20 sequences complementary to the 3z-env probe.
Additional samples include RNA (100 µg) from an
untransfected WHHL rabbit (lane 'Mock') and from WHHL
rabbits injected with LDLR-complex and harvested 10
minutes (lane '10 min'), 4 hours (lane '4 hr'), and
25 24 hours (lanes 24A and 24B, representing two
different animals). The undigested probes (2×10^3
cpm/lane) were electrophoresed in lanes '3Z-env' and
'3Z-wLDLR'. Molecular weight markers (γATP labeled
pBr322/ΦX Hae III fragments) and their corresponding
30 sizes in base pairs are presented in the far right
lane. The closed arrow indicates the location of the
3Z-env protected band while the open arrow indicates
the location of the 3Z-wLDLR protected band.

Endogenous LDL receptor RNA was specifically
detected in RNase protection assays as a 80 bp
protected fragment; no hybridization to human LDL
receptor RNA was noted. The intensity of the
05 resulting bands is proportional to the amount of RNA
used in the initial hybridization suggesting that
this assay can be used to quantify recombinant and
endogenous transcripts.

Recombinant RNA was not detected in
10 mock-transfected liver or in liver harvested 10
minutes after administration of the LDLR-complex.
Livers removed 4 hours and 24 hours after _in vivo_
gene transfer contained significant levels of the
recombinant transcript. Quantitative analysis of the
15 assay was used to estimate the abundance of vector-
derived RNA relative to the endogenous LDL receptor
transcript; levels of the recombinant LDL receptor
transcript rose from undetectable at 10 minutes (<
0.05% of endogenous), to 1.3% of endogenous at 4
20 hours, and 4.0% (24A) and 2.0% (24B) of endogenous at
24 hours.

Metabolic Effects of LDLR-Complex

Experiments were performed to determine the
metabolic effects of hepatocyte directed gene
25 transfer _in vivo_. WHHL rabbits were injected with
LDLR-complex or CAT-complex and analyzed for changes
in total serum cholesterol. Six WHHL rabbits were
entered into a protocol that involved a two treatment
(injection of p9-12alb(h)LDLR (LDLR-complex) or
30 p9-12albCAT (CAT-complex) plasmid; 5.0 mg of total
DNA in a complex/dose), two period, cross-over
design, with repeated measurements of total serum

-22-

cholesterol within each period (Figure 5). The
DNA/protein complex was injected into the marginal
ear vein over a 2 minute time period. Animals A-C
received LDLR-complex on day 0 and CAT-complex on day
05   12, whereas animals D-F received CAT-complex on day 0
and LDLR complex on day 12. Venous samples were
subsequently obtained through a marginal ear vein and
analyzed for total serum cholesterol. P. Trinder
Ann. Clin. Biochem. _12_:226 (1974). The data were
10   analyzed using a repeated measures analysis of
covariance with the mean of the three baseline
measurements as the covariate. B. Jones and M.G.
Kenward _Design and Analysis of Cross-Over Trials_,
Chapman and Hill, 1989.
15   Injection of the LDLR-complex led to an
immediate but transient decrease in total serum
cholesterol by 25 to 30 % of the pretreatment value;
this did not occur in animals injected with the
CAT-complex. There was a statistically significant
20   time trend on these differences (p<0.001) which
reached a maximum value at two days after injection
and then decreased thereafter, becoming
non-significant following the sixth day after
injection. No evidence of carry-over from one
25   experiment to another was noted (p=0.8148). Areas
under the cholesterol time curve were also analyzed.
Treatment with LDLR-complex was associated with a
highly significant decrease in the area under the
cholesterol time curve (p=0.0035) compared to
30   injection of the CAT-complex. There was no evidence
of any carry-over (p=0.1625) or period effects
(p=0.8010).

Equivalents

Those skilled in the art will recognize, or b able to ascertain using no more than routine experimentation, numerous equivalents to the specific
05 procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

-24-

Claims

1.   A soluble molecular complex for targeting a gene
     encoding a cell surface receptor to a specific
     cell, the complex comprising an expressible gene
05   encoding the cell surface receptor complexed
     with a carrier of a cell-specific binding agent
     and a gene-binding agent.

2.   A soluble molecular complex of claim 1, wherein
     the expressible gene is DNA.

10 3.   A soluble molecular complex of claim 1, wherein
     the expressible gene encodes the human LDL
     receptor.

4.   A soluble molecular complex of claim 1, wherein
     the gene-binding agent is a polycation.

15 5.   A soluble molecular complex of claim 4, wherein
     the polycation is polylysine.

6.   A soluble molecular complex of claim 1, wherein
     the cell-specific binding agent binds a surface
     receptor of the cell which mediates endocytosis.

20 7.   A soluble molecular complex of claim 6, wherein
     the cell-specific binding agent is a ligand for
     an asialoglycoprotein receptor.

8. A s luble mol cular c mplex of claim 7, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

05 9. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent by a noncovalent bond.

10. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is linked to the gene-binding agent by a covalent bond.

10 11. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent so that the gene is released in functional form under intracellular conditions.

15 12. A pharmaceutical composition comprising a solution of the molecular complex of claim 1 and a physiologically acceptable vehicle.

13. A soluble molecular complex for targeting a gene encoding a cell surface receptor to a 20 hepatocyte, the complex comprising an expressible gene encoding the cell surface receptor complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

25 14. A soluble molecular complex of claim 13, wherein the expressible gene encodes the human LDL receptor.

-26-

15. A soluble molecular complex of claim 13, wher in
     the polycation is polylysine.

16. A soluble molecular complex of claim 13, wherein
     the gene is contained in an expression vector
05   along with genetic regulatory elements necessary
     for expression of the gene by the hepatocyte.

17. A soluble molecular complex of claim 16, wherein
     the expression vector is a plasmid or viral DNA.

18. A soluble molecular complex for targeting a gene
10   encoding the human LDL receptor to a hepatocyte,
     the complex comprising an expressible gene
     encoding the human LDL receptor complexed with a
     carrier of a ligand for the asialoglycoprotein
     receptor and a polycation.

15   19. A soluble molecular complex of claim 18, wherein
     the polycation is polylysine.

20. A method of delivering an expressible gene
     encoding a cell surface receptor to a specific
     cell of an organism for expression by the cell,
20   comprising administering to the organism a
     soluble molecular complex comprising the
     expressible gene encoding the cell surface
     receptor complexed with a carrier of a
     cell-specific binding agent and a gene-binding
25   agent.

21. A method of claim 20, wherein the expressible
     gene is DNA.

22. A method of claim 20, wherein the expressible
    gene encodes the human LDL receptor.

23. A method of claim 20, wherein the gene-binding
    agent is a polycation.

05 24. A method of claim 23, wherein the polycation is
    polylysine.

25. A method of claim 20, wherein the cell-specific
    binding agent binds a surface receptor of the
    cell which mediates endocytosis.

10 26. A method of claim 25, wherein the cell-specific
    binding agent is a ligand for an
    asialoglycoprotein receptor.

27. A method of claim 26, wherein the ligand is an
    asialoglycoprotein and the targeted cell is a
15   hepatocyte.

28. A method of claim 20, wherein the molecular
    complex is administered intravenously.

29. A method of selectively transfecting hepatocytes
    in vivo with a gene encoding a cell surface
20   receptor, comprising intravenously injecting a
    pharmaceutically acceptable solution of a
    molecular complex comprising an expression
    vector containing an expressible gene encoding
    the cell surface receptor complexed with a
25   carrier of a ligand for the asialoglycoprotein
    receptor and a polycation.

-28-

30. A method of claim 29, wherein the expressible
    gene encodes the human LDL receptor.

31. A method of claim 29, wherein the polycation is
    polylysine.

1 / 5
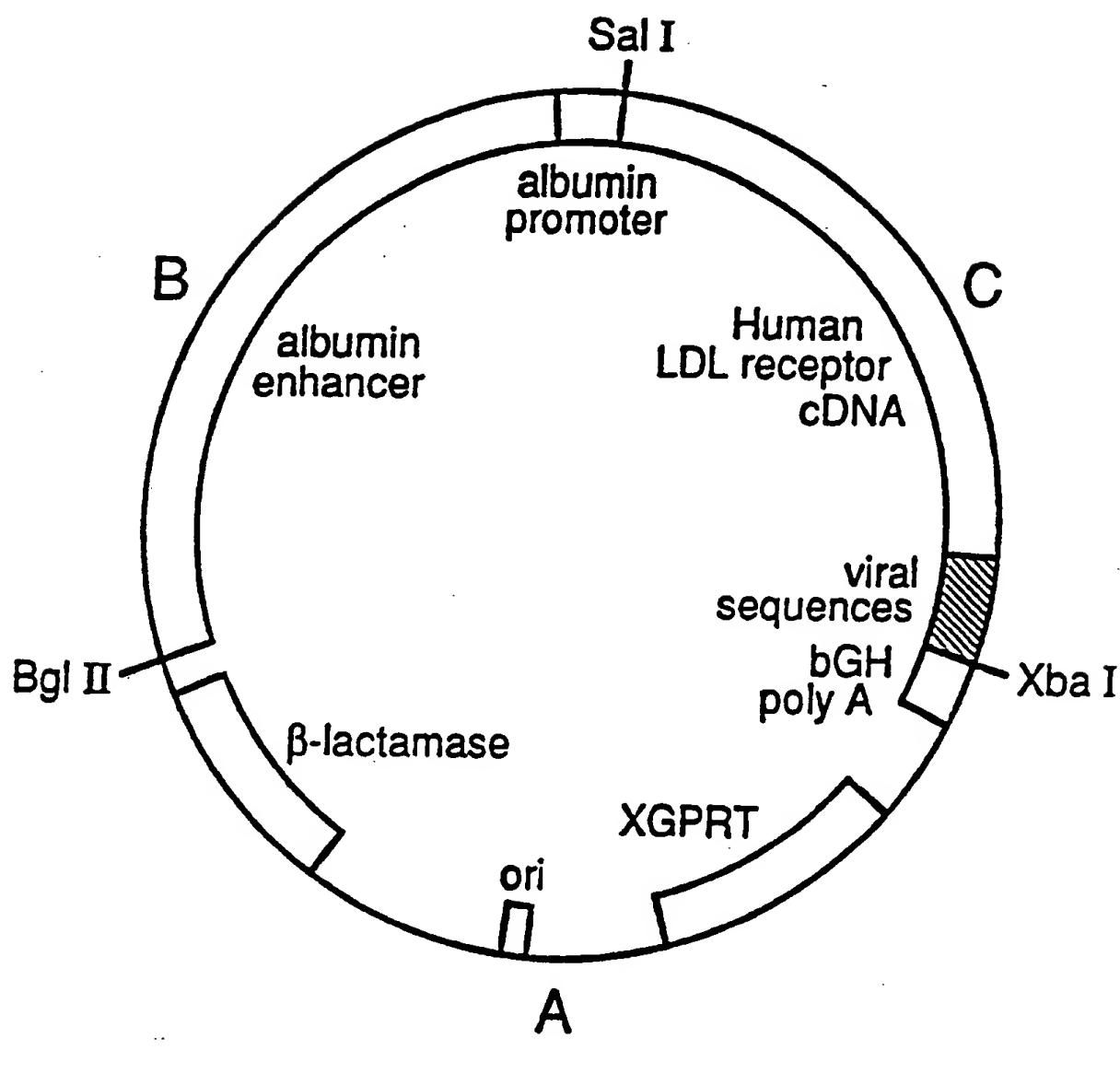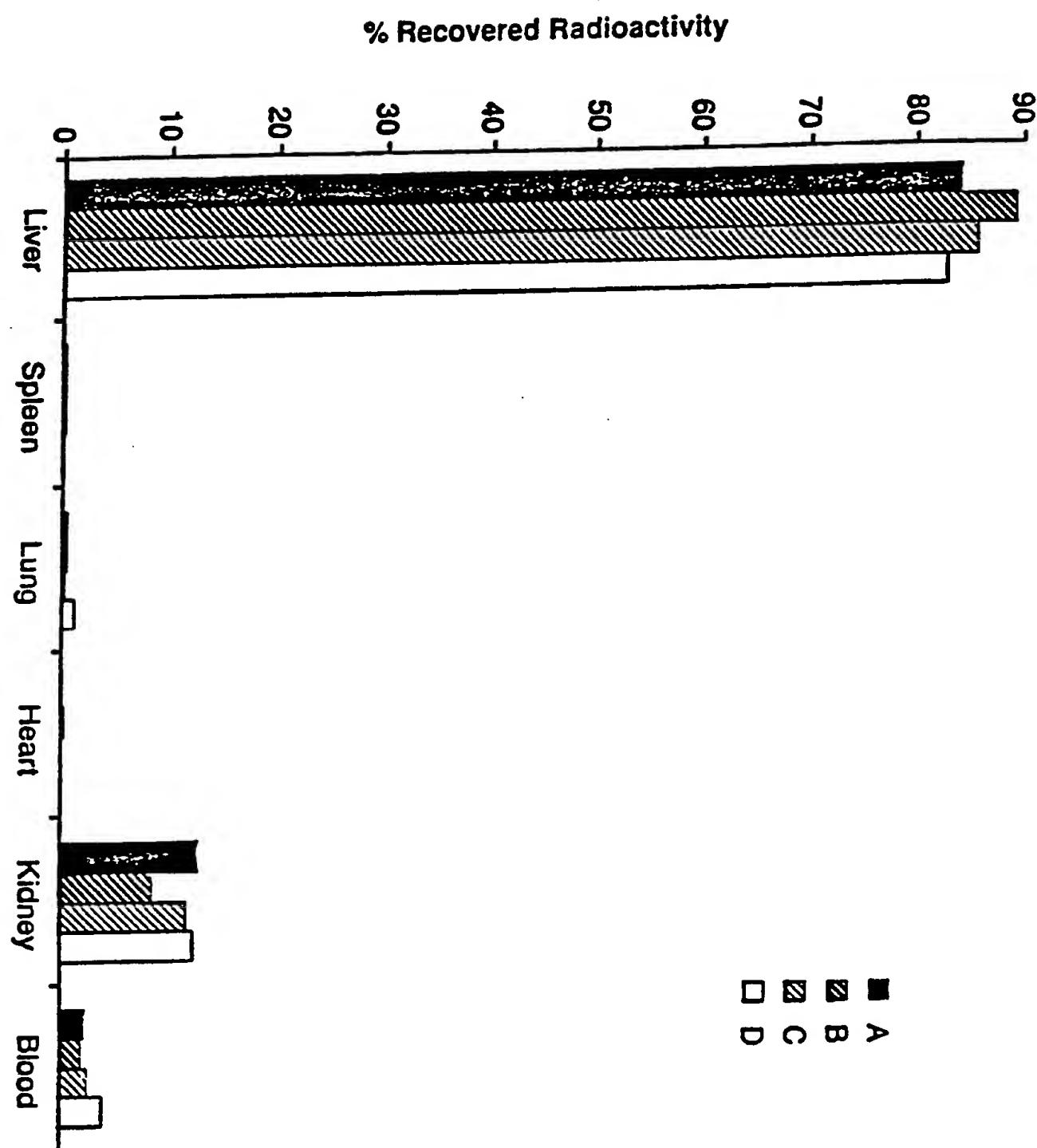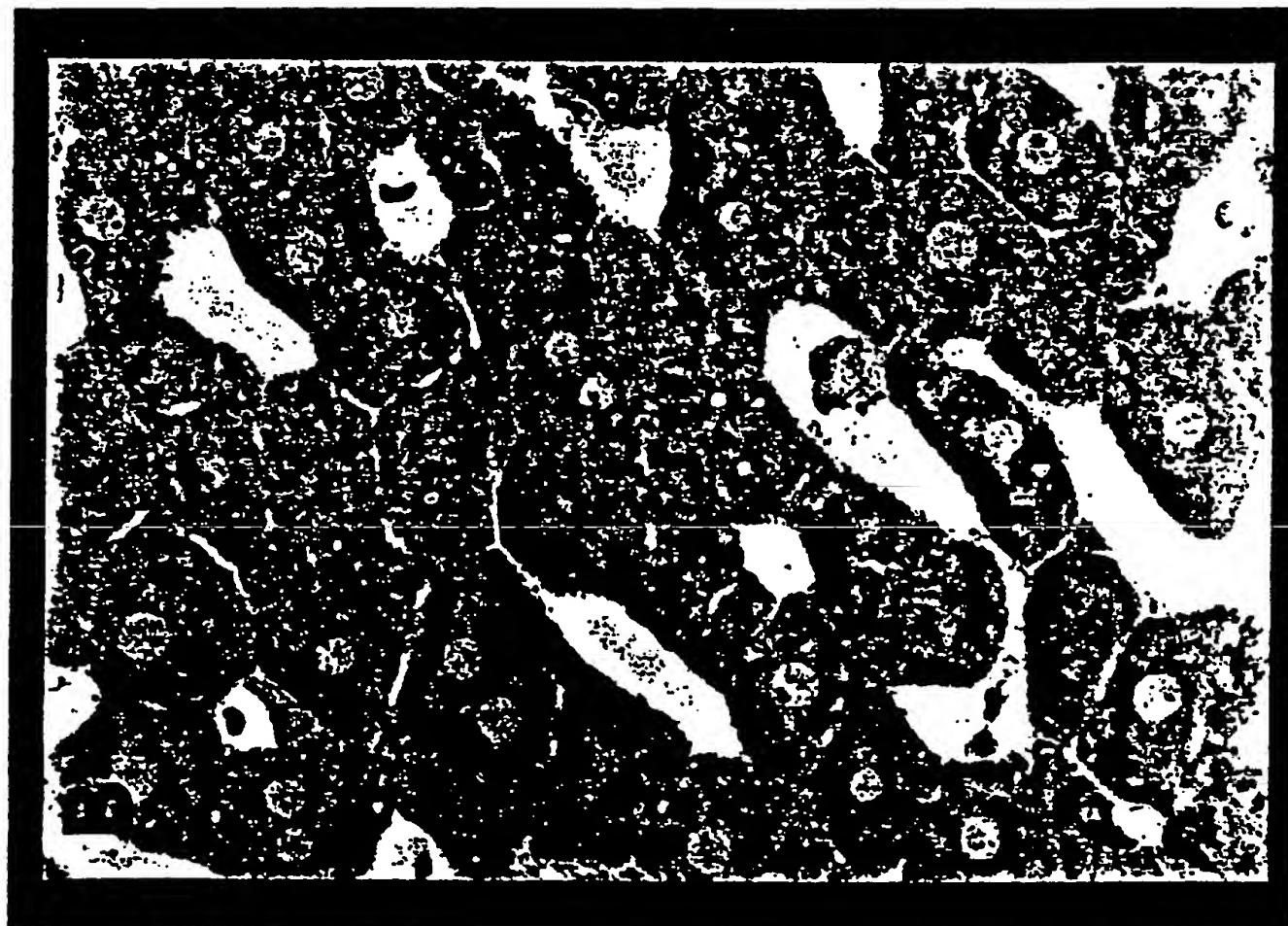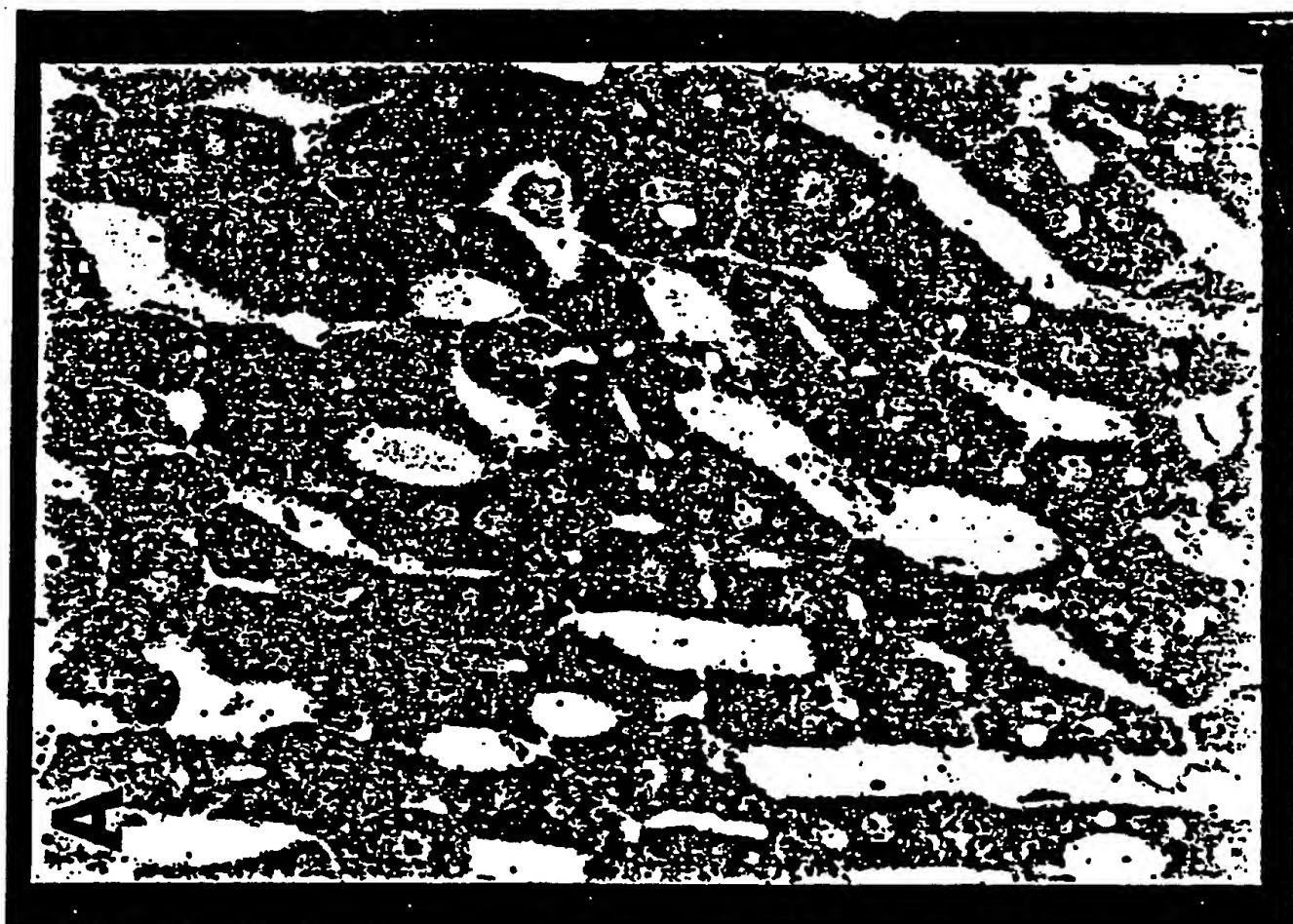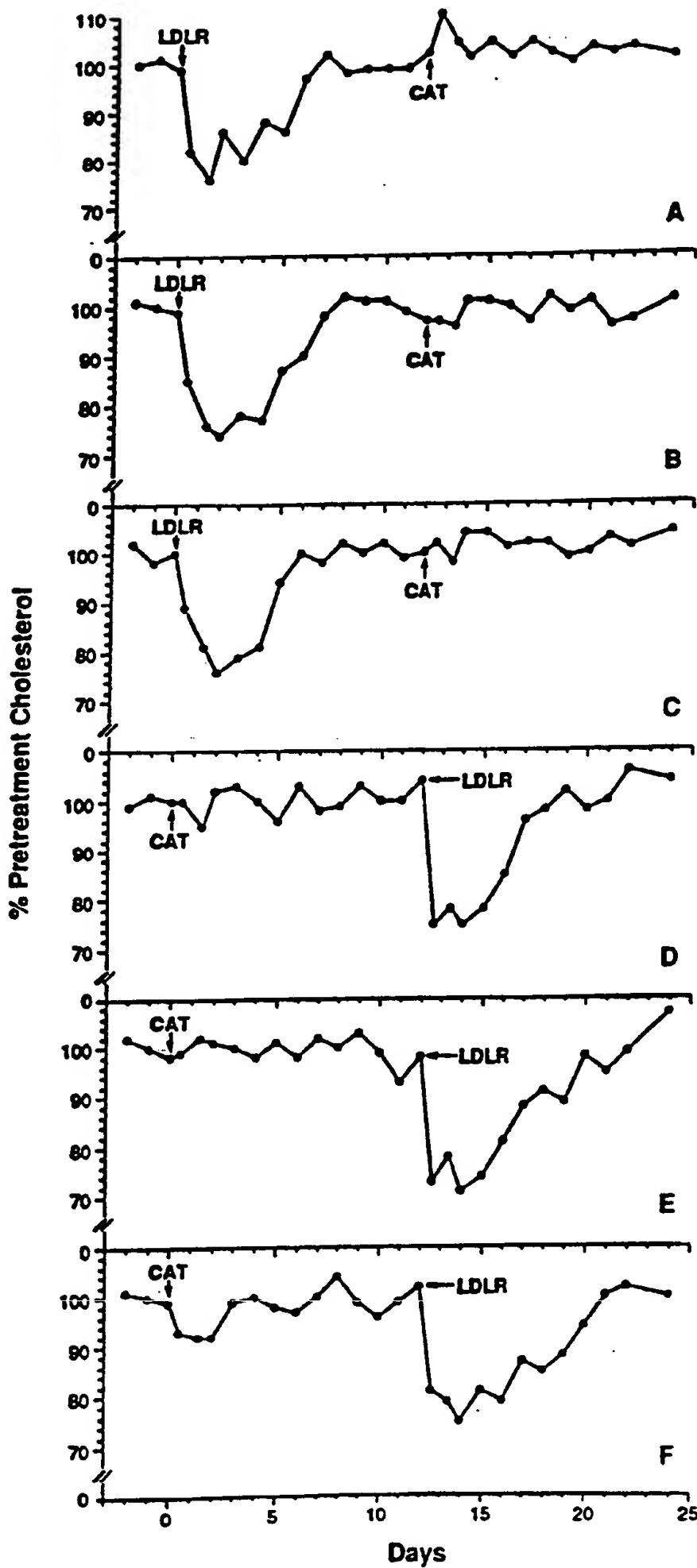
FIGURE 1

% Recovered Radioactivity

FIGURE 2

FIGURE 3

4 / 5

FIGURE 4

FIGURE 5

I. CLASSIFICATION OF SUBJECT MATTER   (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/87;   C12N15/12;   A61K48/00;   C07K15/00

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category°	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No.¹³
X	CLINICAL RESEARCH vol. 39, no. 2, 9 April 1991, THOROFARE NJ, US page 326A; M. GROSSMAN ET AL.: 'Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in LDL receptor deficient rabbits.' see abstract ---	1-3,6-8, 12-14, 16-18, 20-22, 25-27, 29-30
Y	BIOTHERAPY vol. 3, no. 1, January 1991, DORDRECHT, THE NETHERLANDS pages 87 - 95; G. WU ET AL.: 'Delivery systems for gene therapy.' see abstract --- -/--	1-27, 29-31

° Special categories of cited documents : ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 SEPTEMBER 1992	29. 09. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	NOOIJ F.J.M.
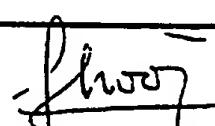
III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 907 136 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 10 August 1989 see claims	1-27, 29-31
P,Y	SOMATIC CELL AND MOLECULAR GENETICS vol. 17, no. 3, May 1991, NEW YORK pages 287 - 301; D. DICHEK ET AL.: 'Low-density-lipoprotein receptors in the Watanabe heritable hyperlipidemic rabbit.' see abstract	1-27, 29-31
Y	HEPATOLOGY vol. 12, no. 4(2), 1990, NEW YORK, US page 922; J. WILSON ET AL.: 'Targeted gene transfer to hepatocytes in vivo: Mechanism(s) of persistent gene expression.' see abstract 338	1-27, 29-31
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 2, 15 January 1992, BALTIMORE MD, US pages 963 - 967; J. WILSON ET AL.: 'Hepatocyte-directed gene transfer in vivo leads to transient improvement of the hypercholesterolemia in low density lipoprotein receptor-deficient rabbits.' see the whole document	1-27, 29-31

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)* incompletely

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   28
because they relate to subject matter not required to be searched by this Authority, namely:

Please see Rule 39.1(iv) - PCT:
Methods for treatment of the human or animal body by surgery or therapy,
as well as diagnostic methods.
./.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/210 (2)

Claims 20-27, 29-31 (searched incompletely)

Although claims 20-27 and 29-31 are directed to a method of treatment of
the animal/human body, the search has been carried out and based on the
alleged effects of the compound/composition.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/09/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8907136	10-08-89	EP-A-	0400047	05-12-90
		JP-T-	3503718	22-08-91